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Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death

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The classical type of programmed cell death is characterized by its dependence on *de novo* RNA and protein synthesis and morphological features of apoptosis. We confirmed that stimulated 2B4.11 (a murine T-cell hybridoma) and interleukin-3 (IL-3)-deprived LyD9 (a murine haematopoietic progenitor cell line) died by the classical type of programmed cell death. Assuming that common biochemical pathways might be involved in the deaths of 2B4.11 and LyD9, we isolated the PD-1 gene, a novel member of the immunoglobulin gene superfamily, by using subtractive hybridization technique. The predicted PD-1 protein has a variant form of the consensus sequence found in cytoplasmic tails of signal transducing polypeptides associated with immune recognition receptors. The PD-1 gene was activated in both stimulated 2B4.11 and IL-3-deprived LyD9 cells, but not in other death-induced cell lines that did not show the characteristic features of the classical programmed cell death. Expression of the PD-1 mRNA in mouse was restricted to the thymus and increased when thymocyte death was augmented by *in vivo* injection of anti-CD3 antibody. These results suggest that activation of the PD-1 gene may be involved in the classical type of programmed cell death.

Key words: apoptosis/growth factor deprivation/immunoglobulin superfamily/programmed cell death/subtractive hybridization

Introduction

Developmentally and physiologically controlled cell deaths can be observed in almost all tissues of various animals (Truman, 1984; Ellis *et al.*, 1991; Golstein *et al.*, 1991; Oppenheim, 1991). Such cell deaths are generally considered 'programmed' and distinguished from 'accidental' deaths that occur by pathological mechanisms (Lockshin and Zakeri, 1991). Most of the cells undergoing programmed death have been shown to require *de novo* synthesis of RNA and protein. Examples are: the death of the intersegmental muscle of the moth *Manduca sexta* after its metamorphosis (Schwartz *et al.*, 1990), the metamorphotic cell death in the tadpole tail (Tata, 1966) and the neuronal death in the chick embryo (Oppenheim *et al.*, 1990). In all of these cases, cell death can be blocked by the inhibitors of macromolecular synthesis, such as actinomycin D or cycloheximide. There are also the *in vitro* experiments showing the inhibitory effect of actinomycin D or cycloheximide on the death of nerve growth factor (NGF)-deprived rat neurons (Martin *et al.*, 1988) and that of mouse thymocytes induced by glucocorticoids (Cohen and Duke, 1984) or by an endogenous superantigen (MacDonald and Lees, 1990). These facts suggest that at least a few genes, if not specific ones, must be expressed to cause programmed cell death.

The term 'apoptosis', on the other hand, is used to describe the morphological characteristics of a class of cell death (Kerr and Harmon, 1991). In cells dying by apoptosis, the chromatin condenses around the periphery of the nucleus, while the mitochondria and other organelles are unaffected. A unique biochemical feature of apoptotic cells includes fragmentation of DNA into oligonucleosomal pieces. Apoptosis is often associated with programmed cell death, but some of the cells undergoing programmed death apparently do not show the characteristic features of apoptosis (Lockshin and Zakeri, 1991). In addition, there are apoptotic cell deaths that can be induced in the absence of any macromolecular synthesis (Golstein *et al.*, 1991). Thus, it is important to note that apoptosis is not synonymous with programmed cell death.

Here we refer to the apoptotic cell death that is dependent on *de novo* synthesis of RNA and protein as the classical type of programmed cell death. A well-known case included in this type is the death of self-reactive immature T lymphocytes (Smith *et al.*, 1989; MacDonald and Lees, 1990). Cells triggered for the classical type of programmed death are likely to determine their fate by turning on 'death-inducing' genes. In order to isolate such genes, it is essential to establish the experimental system in which the classical type of programmed cell death can be easily induced. Four murine cell lines shown below, which can die by apoptosis upon stimulation, are good candidates to be used for the purpose. Like immature T lymphocytes, a T-cell hybridoma 2B4.11 (Ashwell *et al.*, 1987) dies when it is stimulated with its cognate antigen (I-E^k + pigeon cytochrome *c*) or with a combination of ionomycin and phorbol 12-myristate 13-acetate (PMA) (Ucker *et al.*, 1989). An immature B-cell line WEHI-231 (Lanier and Warner, 1981) can be induced to die when its surface IgM molecules are crosslinked by anti-IgM antibodies (Benhamou *et al.*, 1990; Hasbold and Klaus, 1990). A lymphoid/myeloid progenitor cell line LyD9 (Palacios *et al.*, 1987; Kinashi *et al.*, 1988) and a cytotoxic T-cell line CTLL-2 (Gillis and Smith, 1977) require interleukin-3 (IL-3) and interleukin-2 (IL-2), respectively, for their survival and proliferation, and the two cell lines die upon deprivation of the growth factors from the culture.

In this report, we examined whether *de novo* RNA synthesis is required for the apoptotic cell deaths of the above four cell lines and confirmed that it is required for the deaths of 2B4.11 and LyD9, but for that of WEHI-231 or CTLL-2. Assuming that common biochemical pathways might be involved in the classical programmed deaths of

2B4.11 and LyD9 cells, we attempted to identify genes that are specifically expressed in both stimulated 2B4.11 and IL-3-deprived LyD9 cells by using the subtractive hybridization technique. We thus isolated and characterized a programmed cell death-specific cDNA termed PD-1, a novel member of the immunoglobulin gene superfamily. The expression of the PD-1 mRNA was restricted to the thymus and increased when cell death in the thymus was induced by perturbation of the T-cell receptor (TCR)−CD3 complex on thymocytes.

Results

Characterization of apoptotic deaths of 2B4.11, WEHI-231, LyD9 and CTLL-2

To examine whether RNA synthesis is required for the apoptotic deaths of 2B4.11, WEHI-231, LyD9 and CTLL-2, we first aimed to determine the toxic doses of transcriptional inhibitors for the four cell lines. Although actinomycin D (70−200 ng/ml) was not toxic to 2B4.11 and LyD9 (Figure 1C and D), it was highly toxic to both WEHI-231 (Figure 1A, lanes 1−5) and CTLL-2 (Figure 1B, lanes 1−4), and as little as 2−3 ng/ml of actinomycin D induced apoptotic deaths in the two cell lines without any other stimulation. Thus, we avoided using actinomycin D on WEHI-231 or CTLL-2 and tested another transcriptional inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). As shown in Figure 1A (lanes 8−13) and B (lanes 7−10), DRB (<20 μM) was less toxic than actinomycin D to WEHI-231 and CTLL-2.

We then examined the effect of the transcriptional inhibitors on the apoptotic deaths of the four cell lines. When 2B4.11 cells were stimulated for 6 h with a combination of ionomycin and PMA, the characteristic DNA fragmentation of apoptotic cell death appeared (Figure 1C, lane 2). This fragmentation was almost completely suppressed by the

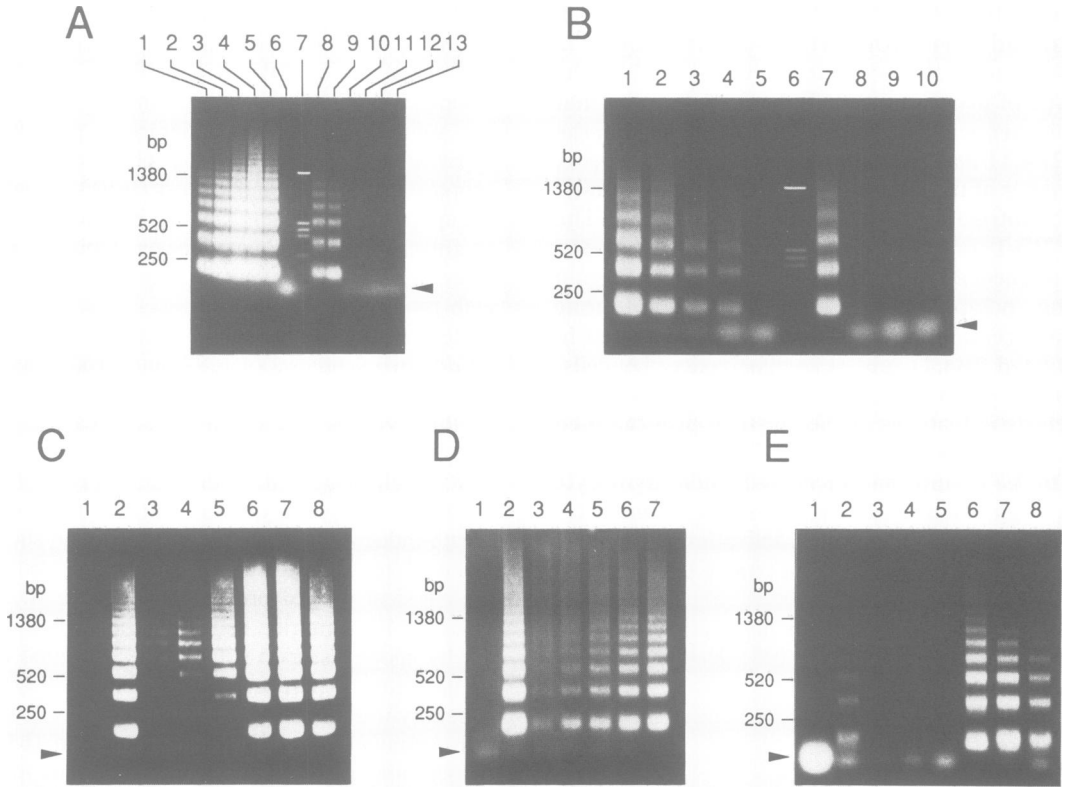

Fig. 1. Effects of transcriptional and translational inhibitors on the apoptotic deaths of 2B4.11, WEHI-231, LyD9 and CTLL-2. (**A**) Toxicity of actinomycin D and DRB to WEHI-231. WEHI-231 cells (5×10^6) were cultured for 12 h in the presence of actinomycin D (lanes 1−5) or DRB (lanes 8−13) and DNA fragmentation was analysed by agarose gel electrophoresis. Actinomycin D was added to 50 ng/ml (lane 1), 25 ng/ml (lane 2), 12.5 ng/ml (lane 3), 6.3 ng/ml (lane 4) and 3.1 ng/ml (lane 5). DRB was added to 180 μM (lane 8), 60 μM (lane 9), 20 μM (lane 10), 6.7 μM (lane 11), 2.2 μM ((lane 12) and 0.7 μM (lane 13). Non-treated WEHI-231 cells were also analysed (lane 6). Lane 7 contained the DNA size markers. (**B**) Toxicity of actinomycin D and DRB to CTLL-2. CTLL-2 cells (5×10^6) were cultured (+IL-2) for 12 h in the presence of actinomycin D (lanes 1−4) or DRB (lanes 7−10), and DNA fragmentation was analysed. Actinomycin D was added to 60 ng/ml (lane 1), 20 ng/ml (lane 2), 6.7 ng/ml (lane 3) and 2.2 ng/ml (lane 4). DRB was added to 60 μM (lane 7), 20 μM (lane 8), 6.7 μM (lane 9) and 2.2 μM (lane 10). Non-treated CTLL-2 cells were also analysed (lane 5). Lane 6 contained the DNA size markers. (**C**) Inhibitory effect of actinomycin D on the stimulation-induced death of 2B4.11. 2B4.11 cells (5×10^6) were cultured for 6 h in the absence (lane 1) or presence (lanes 2−8) of stimulators (500 ng/ml of ionomycin and 10 ng/ml of PMA) and DNA fragmentation was analysed. Actinomycin D (200 ng/ml) was added at 0 h (lane 3), 1 h (lane 4), 2 h (lane 5), 3 h (lane 6), 4 h (lane 7) or 5 h (lane 8) after the stimulation. (**D**) Inhibitory effect of actinomycin D on the IL-3 deprivation-induced death of LyD9. LyD9 cells (5×10^6) were cultured for 12 h in the presence (lane 1) or absence (lanes 2−7) of IL-3 and DNA fragmentation was analysed. Actinomycin D (70 ng/ml) was added at 0 h (lane 3), 2 h (lane 4), 4 h (lane 5), 6 h (lane 6) or 8 h (lane 7) after the IL-3 deprivation. (**E**) Effects of DRB and cycloheximide on the anti-IgM antibody-mediated death of WEHI-231. WEHI-231 cells (5×10^6) were cultured for 23 h in the absence (lane 1) or presence (lanes 2−8) of anti-IgM antibody (10 μg/ml) and DNA fragmentation was analysed. Cycloheximide (lanes 3−5) or DRB (lanes 6−8) was also added at the start of the culture. Cycloheximide was added to 400 ng/ml (lane 3), 133 ng/ml (lane 4) or 44 ng/ml (lane 5). DRB was added to 60 μM (lane 6), 20 μM (lane 7) or 6 μM (lane 8). In (A)−(E), positions of degraded RNA are indicated by arrowheads at the bottom of the gel.

addition of actinomycin D at the time of stimulation (Figure 1C, lane 3). This clearly indicates that *de novo* synthesis of RNA is required for the stimulation-induced death of 2B4.11. To determine when this RNA synthesis occurs, actinomycin D was added at various time points after the stimulation of 2B4.11, and the incubation continued until 6 h after the stimulation. When actinomycin D was added at 1 h after the stimulation, it could still suppress the fragmentation almost completely (Figure 1C, lane 4). However, the addition of actinomycin D at 2 h after the stimulation could only partially suppress the fragmentation (Figure 1C, lane 5) and the suppression was no longer observed when actinomycin D was added 3, 4 and 5 h after the stimulation (Figure 1C, lanes 6–8). This indicates that the synthesis of mRNA required for the cell death is completed 3 h after the stimulation.

The same kind of experiment was also carried out for the LyD9 cells. When IL-3 was depleted from the culture of LyD9 for 12 h, its DNA was digested into oligonucleosomal fragments (Figure 1D, lane 2). Again this fragmentation was almost completely suppressed by adding actinomycin D from the start of IL-3 deprivation (Figure 1D, lane 3). However, the later that actinomycin D was added, the weaker the suppression became (Figure 1D, lanes 4–7). The addition of actinomycin D at 8 h after the deprivation could only partially, but still significantly, suppress the fragmentation (Figure 1D, lane 7). This result suggests that the RNA synthesis required for the death of LyD9 continues for relatively longer periods of time when compared with the death of 2B4.11.

When WEHI-231 cells were stimulated for 23 h with an anti-IgM antibody, the cells began to die by apoptosis (Figure 1E, lane 2). Interestingly, the cell death could not be suppressed by DRB (6–60 μM) at all, but rather enhanced (Figure 1E, lanes 6–8). A lower concentration of DRB (<6 μM) showed neither enhancement nor suppression of the apoptotic death of WEHI-231 (data not shown). On the other hand, cycloheximide could effectively block the DNA fragmentation in stimulated WEHI-231 (Figure 1E, lanes 3–5). These results suggest that *de novo* synthesis of protein, but not RNA, is required for the anti-IgM antibody-mediated death of WEHI-231. Basically the same results were obtained from the experiments on CTLL-2 (data not shown).

Isolation of cDNA clones for mRNAs induced upon programmed cell death

As *de novo* RNA synthesis is undoubtedly required for the apoptotic deaths of 2B4.11 and LyD9, but not for WEHI-231 or CTLL-2, we assumed that mRNAs involved in the classical type of programmed cell death might be expressed in both stimulated 2B4.11 and IL-3-deprived LyD9 cells. The strategy to isolate cDNA clones for such mRNAs is shown in Table I. The probe cDNA was synthesized using mRNA extracted from dying LyD9 cells that had been deprived of IL-3 for 4–6 h. The cDNA was subtracted twice with an excess amount of mRNA extracted from healthy LyD9 cells supplemented with IL-3, and then labelled with ^{32}P by random priming (see Materials and methods for details). To construct a cDNA library, cDNA was synthesized using mRNA extracted from dying 2B4.11 cells that had been stimulated with ionomycin and PMA for 2–3 h. The cDNA was subtracted once with an excess amount of healthy LyD9 mRNA, back-hybridized to an excess amount of stimulated 2B4.11 mRNA, converted to double strand cDNA and cloned into λgt10 vector. We did not use mRNA of unstimulated 2B4.11 in the subtraction step because sometimes the DNA of 2B4.11 is slightly but significantly fragmented even in the absence of any stimulation (Y.Ishida, unpublished observation). We suspected that expression of some 'death genes' might be leaky in 2B4.11 cells and the subtraction between stimulated and unstimulated 2B4.11 cells might eliminate such genes.

The cDNA library constructed as outlined above was expected to contain at least three groups of cDNAs: (i) cDNAs for T cell-specific molecules such as T-cell antigen receptor subunits, (ii) cDNAs for activated T cell-specific molecules such as IL-2, and (iii) cell death-specific cDNAs. Since the cDNA probe prepared as above should not contain the cDNAs belonging to groups (i) and (ii), only the cell death-specific cDNAs that are common between stimulated 2B4.11 and IL-3-deprived LyD9 cells should be identified in our strategy.

The subtracted probe was 25-fold enriched for the IL-3 deprivation-specific sequences as judged by the fact that 96% of the cDNA mass was removed in the subtraction step. On the other hand, the subtracted library was 10-fold enriched because 90% of the cDNA mass was subtracted. About 20 000 clones in the library were screened using the subtracted probe and four strongly positive clones with different insert lengths were obtained. The restriction maps of the four clones overlapped and the four clones hybridized to one another even under a very stringent condition. The gene, from which the four cDNAs were derived, was referred to as PD-1. Another clone with a longer insert (PD-1 cDNA #7) was obtained from an ordinary cDNA library of stimulated 2B4.11 mRNA.

Structure of PD-1 cDNA

The nucleotide sequence of PD-1 cDNA #7 and its predicted amino acid sequence are shown (Figure 2A) together with the schematic representation and restriction map of the clone (Figure 2B). The longest open reading frame (ORF) encodes a putative PD-1 protein of 288 amino acids. The location of the initiation codon is verified by the presence of an in-frame stop codon upstream of the reading frame. The PD-1 protein contains two hydrophobic regions, one at the N terminus and the other in the middle, which are likely to serve as a signal peptide and a transmembrane segment, respectively. Comparison of the N-terminal sequence of the PD-1 protein with typical signal peptide cleavage sites (von

Table I. Strategy of subtractive hybridization

Subtracted cDNA probe		Subtracted cDNA library	
Step	Procedure	Step	Procedure
1	Withdrawal of IL-3 from LyD9	1	Stimulation of 2B4.11 with ionomycin and PMA
2	Extraction of mRNA	2	Extraction of mRNA
3	First strand synthesis of cDNA	3	First strand synthesis of cDNA
4	Two successive subtractions with LyD9 mRNA	4	Subtraction with LyD9 mRNA
5	Labelling with ^{32}P by random priming	5	Back-hybridization to stimulated 2B4.11 mRNA
6	Use as probe	6	Second strand synthesis of cDNA
		7	Cloning into λgt10 library

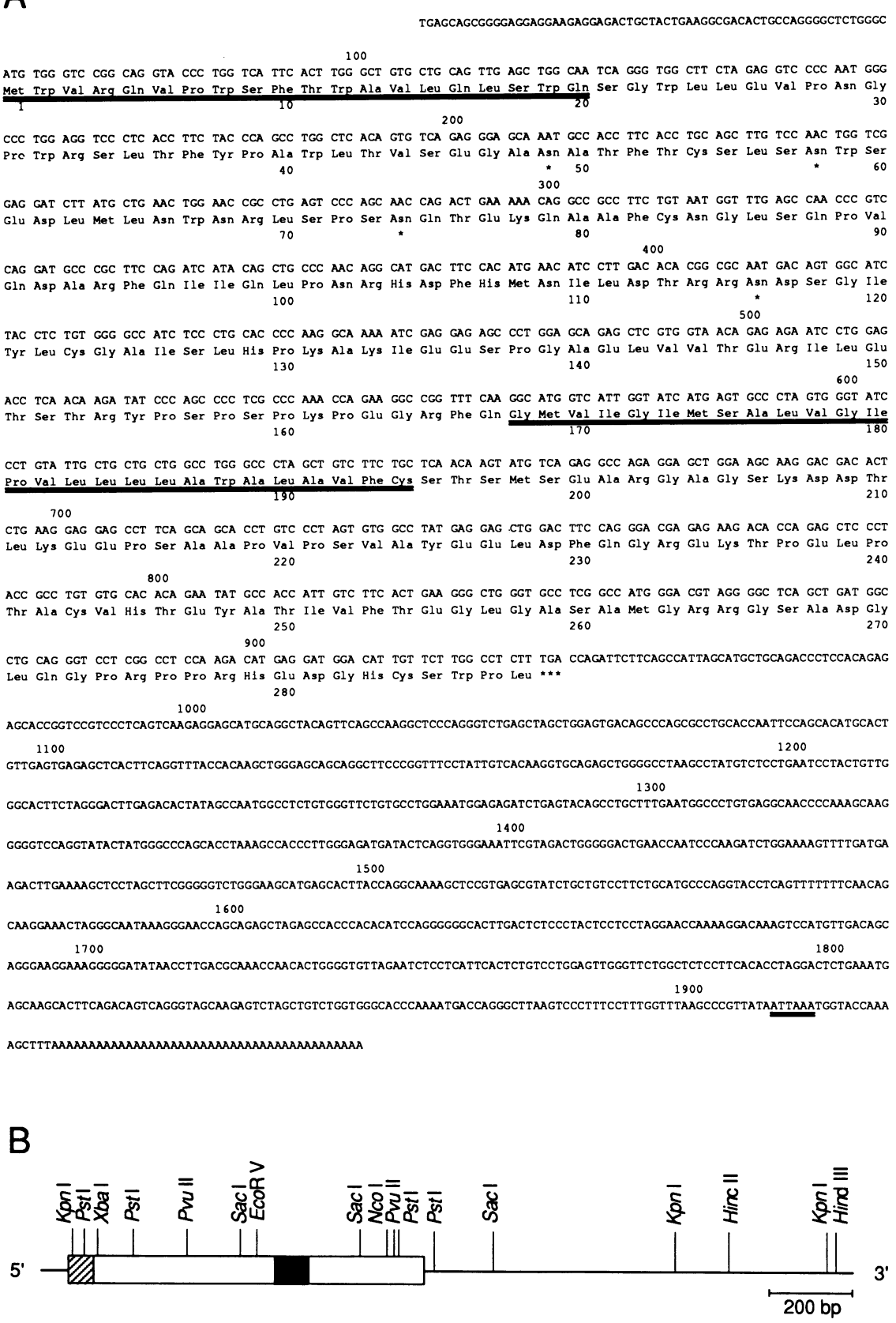

Fig. 2. Structure of murine PD-1 cDNA. (**A**) Nucleotide and predicted amino acid sequences of the murine PD-1 cDNA #7. Numbers above and below each line refer to the nucleotide and amino acid positions, respectively. Amino acids are numbered from the putative translational initiation site (Met1). The signal sequence, transmembrane segment and poly(A) addition signal are underlined. Four potential *N*-glycosylation sites are indicated by asterisks. (**B**) Schematic representation and restriction map of the PD-1 cDNA #7. The box represents the ORF. The hatched and closed boxes indicate the signal sequence and the transmembrane segment, respectively.

Heijne, 1986) suggests that the signal peptide would be cleaved after Gln20. Thus the predicted mature form of the PD-1 protein would contain 268 amino acids and consist of an extracellular domain (147 amino acids), a transmembrane region (27 amino acids) and a cytoplasmic domain (94 amino acids). Four potential *N*-glycosylation sites are found in the putative extracellular domain.

Comparison of the amino acid sequence of the PD-1 protein with all sequences present in the National Biomedical Research Foundation data base revealed that the extracellular domain of the PD-1 protein is homologous to some members of the immunoglobulin superfamily (Figure 3A). Immunoglobulin domains have been classified into V, C1 and C2 sets based on the conserved amino acid patterns and the number of antiparallel β-strands (Williams and Barclay, 1988). The 68 amino acid residues between two cysteine residues (Cys54 and Cys123) in PD-1 bear resemblance to a disulfide-linked immunoglobulin domain of the V-set sequences. In addition, all of the four amino acid residues characteristic of many V-set sequences are also conserved in PD-1 (Arg94, Phe95, Asp117 and Gly119).

The cytoplasmic domain of the predicted PD-1 protein contains a variant form of the consensus sequence (D/E-X_8-D/E-X_2-Y-X_2-L/I-X_7-Y-X_2-L/I) found in the

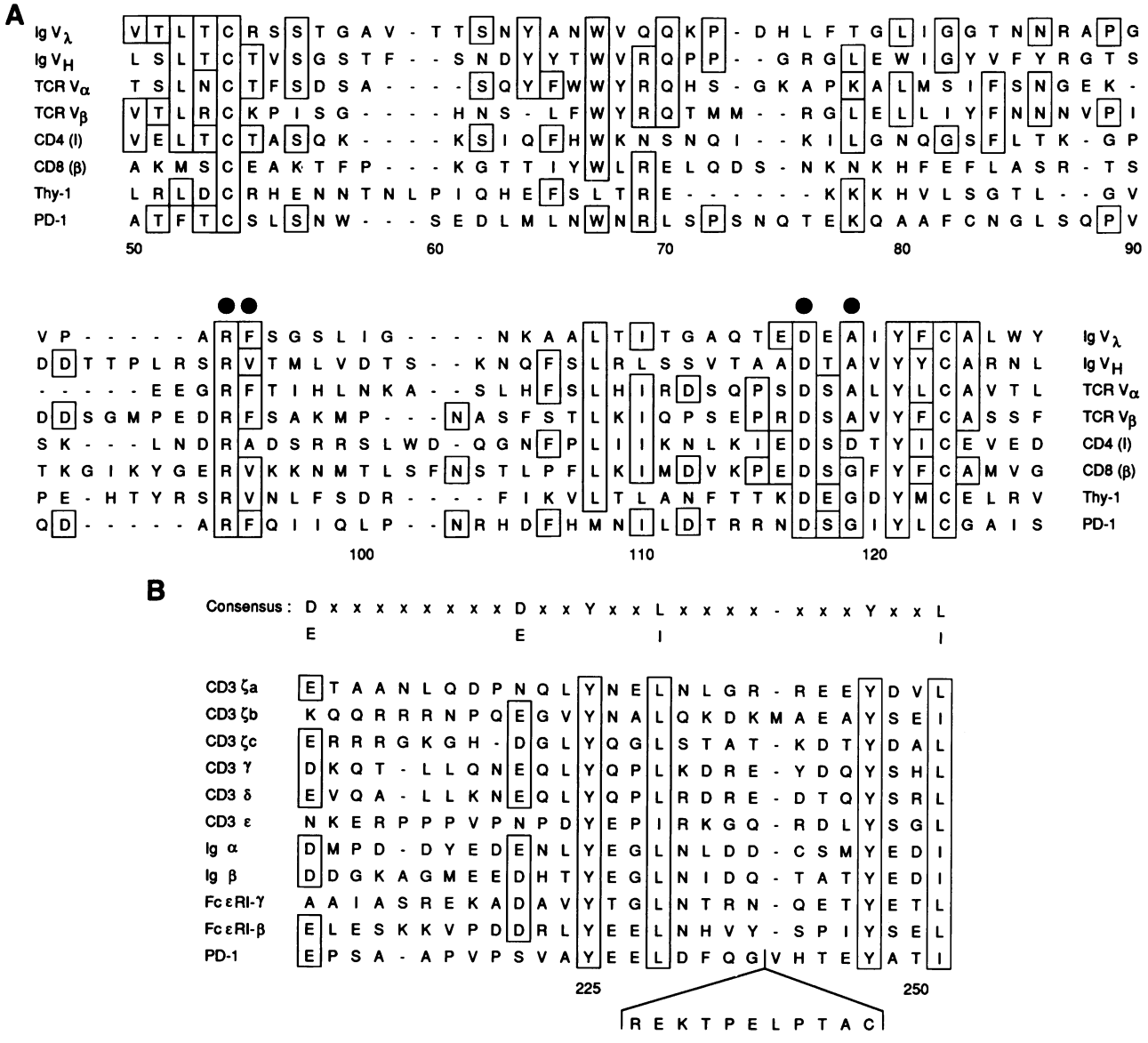

Fig. 3. Homology of PD-1 with other cell surface molecules in the immune system. (**A**) Comparison of the extracellular-domain sequences of PD-1 and several members of the immunoglobulin superfamily. All the seven sequences aligned for the comparison with PD-1 are classified as V-set sequences (Williams and Barclay, 1988). Boxed residues are conserved among more than three out of eight sequences. Characteristic residues among many V-set sequences are indicated by closed circles. IgV_λ, mouse immunoglobulin (Ig) λ light chain variable (V) region; IgV_H, human Ig heavy chain V region; TCR V_α, mouse TCR α chain V region; TCR V_β, human TCR β chain V region; CD4 (I), human CD4 domain I; CD8 (β), rat CD8 β chain; Thy-1, rat Thy-1. The sequences shown here are taken from Williams and Barclay (1988). (**B**) Comparison of the intracellular-domain sequences of PD-1 and murine polypeptides associated with antigen receptors and Fc receptors. Each of CD3 ζa, CD3 ζb and CD3 ζc sequences is found in a single CD3 ζ polypeptide. All the sequences aligned for the comparison with PD-1 are taken from Wegener *et al.* (1992). Residues identical to the consensus sequence are boxed. Extra 11 amino acids inserted between Gly232 and Val244 in PD-1 are shown below. In (A) and (B), amino acid residues are shown in the single letter and gaps are represented by dashes. Numbers below the alignments refer to the amino acid positions of PD-1 shown in Figure 2A.

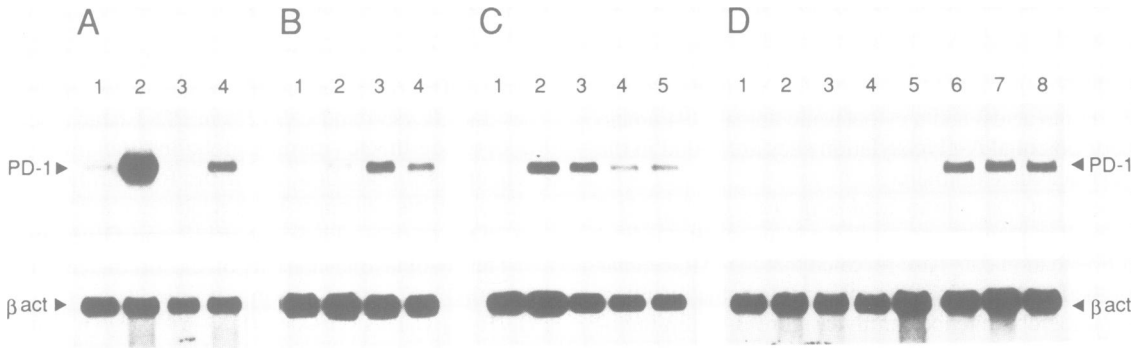

Fig. 4. Northern blot analysis of murine PD-1 mRNA. (**A**) Induction of the PD-1 mRNA in stimulated 2B4.11 and IL-3-deprived LyD9 cells. 3 μg of poly(A)$^+$ RNA prepared from 2B4.11 cells (lane 1), 2B4.11 cells stimulated for 2−3 h with ionomycin (500 ng/ml) and PMA (10 ng/ml) (lane 2), LyD9 cells (lane 3) or LyD9 cells deprived of IL-3 for 4−6 h (lane 4), was electrophoresed in a 1.2% agarose gel and analysed by Northern hybridization as described in Materials and methods. The Northern filter was rehybridized with the mouse β-actin cDNA probe. (**B**) Kinetic analysis of the PD-1 mRNA expression in stimulated 2B4.11 cells. 2B4.11 cells were unstimulated (lane 1) or stimulated as in (A) for 1 h (lane 2), 3 h (lane 3) or 5 h (lane 4). 3 μg of poly(A)$^+$ RNA was prepared from the cells and analysed as in (A). (**C**) Kinetic analysis of the PD-1 mRNA expression in IL-3-deprived LyD9 cells. LyD9 cells were cultured in the presence (lane 1) or absence of IL-3 for 3 h (lane 2), 6 h (lane 3), 9 h (lane 4) or 12 h (lane 5). 3 μg of poly(A)$^+$ RNA was prepared from the cells and analysed as in (A). (**D**) Expression of the PD-1 mRNA in other cell lines. 3 μg of poly(A)$^+$ RNA was prepared from WEHI-231 cells (lane 1), WEHI-231 cells stimulated with anti-IgM antibody (10 μg/ml) for 14 h (lane 2), CTLL-2 cells (lane 3), CTLL-2 cells deprived of IL-2 for 6 h (lane 4), L929 fibroblasts (lane 5), WR19L cells (lane 6) or WR19L cells treated with TNF-α (15 ng/ml) for 1.5 h (lane 8) or 3 h (lane 8). Northern hybridization was carried out as in (A).

cytoplasmic tails of most of the polypeptides associated with antigen receptors and Fc receptors (Reth, 1989; Figure 3B). It was recently shown that a single unit of this consensus sequence is sufficient to transduce signals (Letourneur and Klausner, 1992; Romeo *et al.*, 1992; Wegener *et al.*, 1992). Athough the number of non-conserved amino acids between Leu228 and Tyr248 in PD-1 is considerably larger than that of the consensus sequence, most of the conserved residues and spacer lengths in the consensus sequence are conserved in the cytoplasmic domain of the predicted PD-1 protein.

Cell death-associated expression of PD-1 mRNA

To know whether the PD-1 gene is activated in both stimulated 2B4.11 and IL-3-deprived LyD9 cells as expected, Northern hybridization was carried out (Figure 4A). We could detect a slight expression of PD-1 mRNA (2.2 kb) in unstimulated 2B4.11 cells, but the expression was strongly augmented (>10-fold) in stimulated 2B4.11 cells (Figure 4A, lanes 1 and 2). On the other hand, only IL-3-deprived, but not IL-3-supplemented LyD9 cells expressed PD-1 mRNA (Figure 4A, lanes 3 and 4).

Next we examined the kinetics of the PD-1 expression in stimulated 2B4.11 and IL-3-deprived LyD9 cells. As shown in Figure 4B, the expression of PD-1 mRNA in stimulated 2B4.11 cells reached its maximum level at ~3 h after stimulation and slightly reduced at 5 h. On the other hand, the PD-1 expression in IL-3-deprived LyD9 cells increased until 3−6 h and gradually decreased around 9−12 h after IL-3 deprivation (Figure 4C). These results are compatible with the actinomycin D suppression profiles of the programmed deaths of 2B4.11 and LyD9 cells (Figure 1C and D).

As expected from the fact that neither actinomycin D nor DRB could suppress the stimulation-induced death of WEHI-231 and IL-2 deprivation-induced death of CTLL-2, no induction of the PD-1 mRNA was observed in these cells upon apoptosis (Figure 4D, lanes 1−4). Interestingly, a mouse T-cell lymphoma line, WR19L (Itoh *et al.*, 1991), whose apoptotic death can be rapidly induced by tumor necrosis factor-α (TNF-α) within 2−3 h of incubation (data not shown), was found to express PD-1 mRNA, but the expression was not augmented at all by the TNF-α treatment (Figure 4D, lanes 6−8). In addition, another TNF-α-sensitive mouse cell line L929 (a fibroblast cell line) did not express PD-1 mRNA (Figure 4D, lane 5). Since TNF-α can mediate cell death in the presence of actinomycin D or cycloheximide (Rubin *et al.*, 1988), it is possible that WR19L and L929 cells treated with TNF-α do not require the induced expression of the PD-1 mRNA for their apoptotic deaths.

To determine the tissue distribution of the PD-1 expression, mRNAs extracted from various mouse tissues were analysed by Northern hybridization. As shown in Figure 5A, the PD-1 mRNA expression was restricted to the thymus where thymocyte death occurs constitutively. No PD-1 mRNA was detected in the brain, heart, lung, spleen, liver or kidney. However, very faint bands could be detected in lanes with mRNA from the spleen and lung after a prolonged exposure (data not shown).

Next we examined whether the expression of the PD-1 mRNA in the thymus could be augmented by enhancing the thymocyte death *in vivo*. When mice are intraperitoneally injected with an anti-CD3 monoclonal antibody (mAb) 145-2C11 (Leo *et al.*, 1987), a significant level of the apoptotic cell death can be induced in the thymus (Shi *et al.*, 1989). Thus, we extracated mRNAs from the thymus and spleen of the mice injected with the anti-CD3 mAb and the PD-1 expression was examined by Northern hybridization (Figure 5B). In the thymus of the mice injected with the anti-CD3 mAb, we observed extensive DNA fragmentation (data not shown) and the expression of the PD-1 mRNA was augmented (~3-fold) at 8 and 12 h after the injection (Figure 5B, lanes 4 and 5). On the other hand, neither DNA fragmentation (data not shown) nor augmentation of the PD-1 expression was observed in the thymus of the phosphate-buffered saline (PBS)-injected mice (Figure 5B, lanes 2 and 3). Interestingly, the expression of the PD-1 mRNA was enhanced not only in the thymus, but also in the spleen of the anti-CD3 mAb-injected mice (Figure 5B, lanes 9 and 10). Again such enhancement was not observed in the spleen

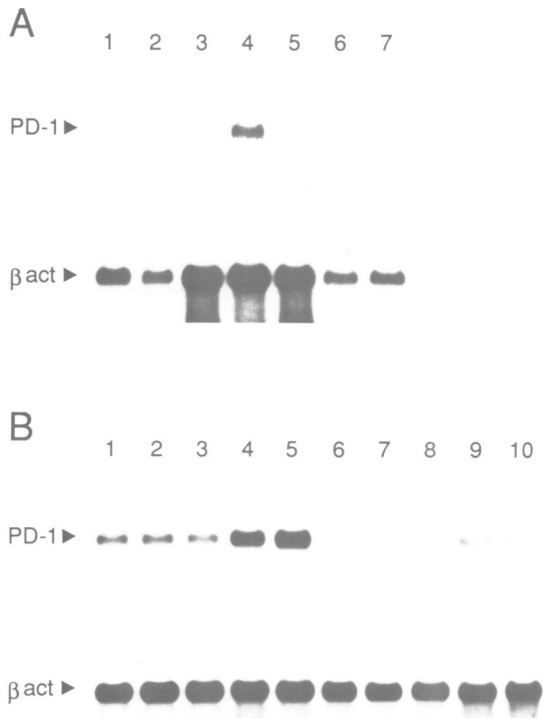

Fig. 5. Cell death-associated expression of murine PD-1 mRNA *in vivo*. (**A**) Tissue distribution of murine PD-1 mRNA. 3 μg of poly(A)$^+$ RNA prepared from the brain (lane 1), heart (lane 2), lung (lane 3), thymus (lane 4), spleen (lane 5), liver (lane 6) or kidney (lane 7), was electrophoresed in a 1.2% agarose gel and analysed by Northern hybridization as described in Materials and methods. The Northern filter was rehybridized with the mouse β-actin cDNA probe. (**B**) Augmented expression of murine PD-1 mRNA in the thymus and spleen of mice injected with anti-CD3 mAb. 3 μg of poly(A)$^+$ RNA was prepared from the normal thymus (lane 1), the thymus of mice injected with PBS(−) (lanes 2 and 3), the thymus of mice injected with anti-CD3 mAb (lanes 4 and 5), the normal spleen (lane 6), the spleen of mice injected with PBS(−) (lanes 7 and 8) or the spleen of mice injected with anti-CD3 mAb (lanes 9 and 10). RNA was prepared at 8 h (lanes 2, 4, 7 and 9) or 12 h (lanes 3, 5, 8 and 10) after the injection. Intensities of the bands of PD-1 mRNA in lanes 6−10 were enhanced by a longer exposure as compared to those in lanes 1−5. Northern hybridization was carried out as in (A).

of the PBS-injected mice (Figure 5B, lanes 7 and 8). The enhanced expression of the PD-1 mRNA in the spleen of the anti-CD3 mAb-injected mice agrees with a weak level of apoptotic cell death detected in the spleen of such mice (data not shown).

Discussion

The PD-1 gene is activated in both of the two different types of lymphoid cell lines (2B4.11 and LyD9) when they are induced to die by the different manipulations (ionomycin/PMA treatment and IL-3 deprivation, respectively). As the only known common feature between stimulated 2B4.11 and IL-3-deprived LyD9 cells is the programmed cell death, it is probable that PD-1 plays a role in the death-inducing process in the two manipulated cell lines. The expression kinetics of the PD-1 mRNA in death-induced 2B4.11 and LyD9 cells also suggests that the PD-1 mRNA may be one of the molecules whose *de novo* synthesis is required for the two cell lines to die. In addition, the restricted expression of the PD-1 mRNA in the mouse thymus and its augmentation by the death-enhancing manipulation further enforce the notion that PD-1 is a cell death-associated gene.

It is likely that some other types of apoptotic deaths are mechanistically different from that of 2B4.11 and LyD9 cells. The anti-IgM antibody-mediated death of WEHI-231 cells and IL-2 deprivation-induced death of CTLL-2 cells could not be suppressed, but rather enhanced by the transcriptional inhibitors, while cycloheximide could effectively block the deaths of the two cell lines. In addition, no induction of the PD-1 mRNA was observed in the deaths of these cells. The results suggest that only *de novo* synthesis of protein but not RNA including the mRNA of PD-1 is required for the deaths of WEHI-231 and CTLL-2 cells. Interestingly, a human oncogene *bcl-2* fails to block the IL-2 deprivation-induced death of CTLL-2 cells (Nuñez *et al.*, 1990), whereas the *bcl-2* expression can inhibit a variety of apoptotic cell deaths (Vaux *et al.*, 1988; Sentman *et al.*, 1991; Strasser *et al.*, 1991). This finding also suggests the possibility that CTLL-2 may employ a unique set of death-inducing mechanisms.

The cell death mediated by TNF-α seems to be another example, as the TNF-α treated cells were shown to require no macromolecular synthesis for their death (Rubin *et al.*, 1988). Although we could detect the PD-1 mRNA in TNF-α-sensitive WR19L cells, the expression was not augmented at all in WR19L cells induced to die by TNF-α. In addition, another TNF-α-sensitive cell line L929 was found to express no PD-1 mRNA. It is therefore probable that WR19L and L929 cells treated with TNF-α can undergo apoptosis without the activation of the PD-1 gene.

The expression of the PD-1 mRNA is remarkably augmented upon induction of the classical type of programmed cell death, but it is apparent that the PD-1 mRNA alone cannot lead cells to die. For example, 2B4.11 and WR19L, both of which express a small but significant amount of the PD-1 mRNA, grow vigorously and several LyD9 transfectants that are forced to express PD-1 mRNA also proliferate just as fast as parental cells (Y.Ishida, unpublished observation). Thus, if PD-1 actually plays an important role in the process of cell-death induction, it should require some additional factors in order to function. As the PD-1 mRNA can encode a membrane-spanning polypeptide containing a possible signal transducing motif in its cytoplasmic domain (Reth, 1989; Letourneur and Klausner, 1992; Romeo *et al.*, 1992; Wegener *et al.*, 1992), there may be some molecules that can associate with the PD-1 product and generate the negative growth signals.

The homozygous *lpr* (lymphoproliferation) mutation in mice induces multiple autoreactive antibodies and the progressive accumulation of a large number of non-malignant TCRαβ$^+$/CD4$^-$/CD8$^-$ T cells in the peripheral lymph nodes (Cohen and Eisenberg, 1991). Recently, the mouse *lpr* gene has been shown to encode the Fas antigen, a cell surface molecule that can mediate apoptosis upon cross-linking (Yonehara *et al.*, 1989; Watanabe-Fukunaga *et al.*, 1992). These facts suggest that the Fas antigen may play an important role at least in a certain type of T-cell death. Since both stimulated 2B4.11 and IL-3-deprived LyD9 cells express mouse Fas mRNA (Y.Ishida, unpublished observations), one might assume that the induced PD-1 product interacts with the Fas antigen on the same cell and triggers the Fas antigen-mediated apoptotic process. However, this is not probable because (i) a series of bone

marrow transplantation experiments indicate that the Fas antigen and its putative ligand (possibly encoded by the *gld* gene, a homozygous mutation of which causes a *g*eneralized *l*ymphoproliferative *d*isorder) are expressed by different cell compartments (Allen *et al.*, 1990) and (ii) the WR19L cell line expresses both PD-1 and Fas mRNAs, but still grows well (Y.Ishida, unpublished observation). Thus, we are inclined to propose that the PD-1 product may have its own ligand other than the Fas antigen. Furthermore, the Fas antigen may not be directly involved in the classical programmed death in the thymus, as we could observe anti-CD3 mAb-induced cell death in the thymus of mice homozygous for the *lpr* mutation and could not detect augmented expression of the Fas mRNA in the thymus of anti-CD3 mAb-injected normal mice (Y.Agata, unpublished observation).

All the results taken together, we hypothesize that PD-1 functions as a cell-death inducer. However, we should also consider other possibilities: (i) the PD-1 product is a marker for phagocytosis whose expression promotes the elimination of the death-determined cells; (ii) the PD-1 product functions in rescuing some cells from a dying cell population; (iii) PD-1 is expressed fortuitously in the programmed cell death-induced cells.

Materials and methods

Cell culture and apoptosis induction

The murine cell lines 2B4.11 (a generous gift from Dr J.D.Ashwell, National Cancer Institute, Bethesda), WEHI-231 (Lanier and Warner, 1981) and WR19L (provided by Dr S.Nagata, Osaka Bioscience Institute, Osaka, Japan) were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). The murine factor-dependent cell lines LyD9 (Palacios *et al.*, 1987; Kinashi *et al.*, 1988) and CTLL-2 (Gillis and Smith, 1977) were cultured in the complete medium containing 0.2% culture supernatant of X63Ag myeloma cells transfected with a murine IL-3 expression vector (Karasuyama and Melchers, 1988) and 500 pM recombinant (r) human IL-2 (kindly provided by Ajinomoto Co. Ltd, Kanagawa, Japan), respectively. The murine fibroblast cell line L929 (a gift from Dr K.Hama, Ono Pharmaceutical Co. Ltd, Osaka, Japan) was cultured in DMEM (Gibco) supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin.

The reagents used for induction of apoptosis were ionomycin (500 ng/ml, Calbiochem), PMA (10 ng/ml, Calbiochem), goat anti-mouse IgM antibody (10 μg/ml, Jackson ImmunoResearch Laboratories) and r-human TNF-α (15 ng/ml, R&D Systems). For deprivation of the growth factors, cells were washed three times with RPMI 1640 and cultured in the complete medium without growth factors. The reagents used for inhibition of apoptosis were actinomycin D-mannitol (Sigma), DRB (Sigma) and cycloheximide (Wako Pure Chemical Industries, Osaka, Japan).

For induction of apoptosis in the thymus, 5 week old ICR mice were injected intraperitoneally with 200 μl of defibrinated ascitic fluid that had been collected from BALB/c *nu/nu* mice inoculated with the hybridoma 145-2C11 that produces anti-mouse CD3 (ε) mAb (Leo *et al.*, 1987). All the mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Detection of DNA fragmentation in agarose gels

Harvested cells ($5 \times 10^6 - 1 \times 10^7$) were centrifuged and washed once with cold PBS(−). The cell pellet was lysed in 600 μl of a buffer consisting of 10 mM Tris−HCl, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, the lysate was centrifuged (13 000 *g*) for 10 min at 4°C in an Eppendorf microfuge. Then, the supernatant (containing RNA and fragmented DNA, but not intact chromatin) was extracted first with phenol and then with phenol−chloroform:isoamyl alcohol (24:1). The aqueous phase was made to 300 mM NaCl and nucleic acids were precipitated with 2 vol of ethanol. The pellet was rinsed with 70% ethanol, air-dried and dissolved in 15 μl of 10 mM Tris−HCl−1 mM EDTA (pH 7.5). After digesting RNA with RNase A (0.6 mg/ml, at 37°C for 30 min), the sample was electrophoresed in a 2% agarose gel with Boyer's buffer (50 mM Tris−HCl, 20 mM sodium acetate, 2 mM EDTA and 18 mM NaCl, pH 8.05). Then, DNA was visualized by ethidium bromide staining.

cDNA libraries

First strand cDNA was synthesized using poly(A)$^+$ RNA extracted from 2B4.11 cells that had been stimulated with ionomycin (500 ng/ml) and PMA (10 ng/ml) for 2−3 h. cDNA synthesis was carried out using two types of primers (oligo dT or random hexanucleotides), actinomycin D (Sigma), a trace amount of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) and murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories) as described (Sambrook *et al.*, 1989). After hydrolysing RNA template with 0.2 N NaOH (at 68°C for 30 min), single-stranded cDNA was hybridized with an excess amount of IL-3-supplemented LyD9 poly(A)$^+$ RNA in 0.5 M sodium phosphate buffer (pH 6.8), 5 mM EDTA, 0.2% sodium dodecyl sulphate (SDS) under mineral oil at 68°C for 26 h. This reaction reached a Cr_0t value (normalized for the effect of the high salt; Britten *et al.*, 1974) of 7800 mol·s/l. Selection for the unhybridized (single-stranded) cDNA was performed with hydroxyapatite (HAP) (Bio-Rad) equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) at 60°C in a water-jacketed column (Bio-Rad). Single-stranded cDNA separated was then back-hybridized to an excess amount of poly(A)$^+$ RNA extracted from stimulated 2B4.11 cells to a Cr_0t value of 8000 mol·s/l. Recovered cDNA:RNA heteroduplex was converted into double-stranded cDNA and cloned in λgt10 as described (Sambrook *et al.*, 1989). An ordinary λgt10 cDNA library of stimulated 2B4.11 mRNA was constructed by the standard procedure (Sambrook *et al.*, 1989).

Subtracted cDNA probe and library screening

cDNA synthesis, solution hybridization and HAP column chromatography were carried out as described for subtracted cDNA library. Briefly, first strand cDNA was synthesized using poly(A)$^+$ RNA extracted from LyD9 cells that had been deprived of IL-3 for 4−6 h. After hydrolysing RNA template, single-stranded cDNA was hybridized with an excess amount of IL-3-supplemented LyD9 poly(A)$^+$ RNA to a Cr_0t value of 8000 mol·s/l. Unhybridized cDNA was selected by HAP column chromatography and the same hybridization and selection were repeated. Then, single-stranded cDNA obtained was radiolabelled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) to high specific activity by random priming (using random hexanucleotides as primers) as described (Sambrook *et al.*, 1989) and used as subtracted LyD9 cDNA probe. Specific activity of this probe was $\sim 4 \times 10^9$ d.p.m./μg.

Plaque hybridizations were performed using replica nitrocellulose filters from plates containing 2×10^4 cDNA clones. Approximately 5×10^8 d.p.m. of subtracted probe was utilized. The hybridization was carried out at 68°C for 27 h in 5 × SSPE (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4), 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin), 120 μg/ml salmon sperm DNA, 10% dextran sulphate and 0.3% SDS. Washing was performed in 2 × SSC (1 × SSC is 150 mM NaCl and 15 mM Na-citrate) at 55°C for 40 min and then in 0.1 × SSC, 0.1% SDS at 68°C for 30 min.

cDNA clones picked up in the primary screening were subjected to the secondary screening involving both subtractive and differential hybridizations. Duplicate nitrocellulose filters prepared from phage isolates were screened with subtracted LyD9 cDNA probe and with ^{32}P-labelled LyD9 cDNA. Four independent clones were picked up that hybridized with the subtracted LyD9 probe, but not with the LyD9 cDNA probe, and their cDNA inserts were subcloned into Bluescript SK plasmid vectors (Stratagene).

RNA analysis and DNA sequencing

Extraction of total cellular RNA, purification of poly(A)$^+$ RNA and Northern hybridization were carried out by standard methods (Sambrook *et al.*, 1989). For probes, the *Pst*I fragment (650 bp) within the coding region of PD-1 cDNA #7 and the *Eco*RI fragment (300 bp) of pMAβ-3′ut, which contains only the 3′ untranslated region (nucleotides 1639−1892) of mouse β-actin cDNA (Tokunaga *et al.*, 1986), were labelled with ^{32}P by random priming. Nucleotide sequence was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using a modified T7 DNA polymerase (United States Biochemical) and [α-^{32}P]dCTP (3000 Ci/mmol, Amersham).

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Note added in proof

The sequence of the PD-1 cDNA is available from the EMBL/GenBank/DDBJ databases under accession number X67914.